# Direct Quantitative Isolation of Monocarbonyl Compounds from Fats and Oils

▶ A quantitative procedure is described for the direct isolation of carbonyl compounds from fats and oils. Carbonyl compounds in the fat are converted to their 2,4-dinitrophenylhydrazones, subsequently freed of fat, and fractionated by adsorption on activated magnesia and partially deactivated alumina. The fat-free monocarbonyl fraction is then separated into classes on magnesia and the members of each class are obtained by column partition chromatography and identified by supplementary techniques. The procedure is ideally suited for small samples of fat, but can be applied to kilogram quantities. Advantages and limitations of the method are discussed.

This paper describes a method for the direct quantitative isolation of monocarbonyl compounds from fats and oils. The method was developed in conjunction with a study on off-flavor development in stored whole milk powder.

Carbonyl compounds are usually isolated from autoxidized fats and oils by some form of distillation, although other methods such as extraction of water-soluble derivatives have been reported (*6, 11, 12*). Over 95% of the carbonyl compounds occurring in many fats and oils are nonvolatile when the usual methods of distillation are used (*9, 10*). The nature and role of these nonvolatile carbonyl compounds had not been elucidated at the onset of this work, although it has been suggested that part of them, at least, might be involved in fat oxidation (*2*).

In the proposed method, distillation and extraction techniques are circumvented, and, theoretically, all carbonyl compounds capable of forming a 2,4-dinitrophenylhydrazone under the conditions outlined are isolated lipide-free, regardless of the amount of starting material.

## EXPERIMENTAL

**Materials.** Seasorb 43 (activated magnesia, Fisher Scientific Co., Silver Spring, Md.) is used as received. Hexane (Phillips' high purity grade) and benzene (ACS grade) are rendered carbonyl-free by the method of Schwartz and Parks (*17*). Celite 545 is dried at 150° C. for 24 hours. Nitromethane (Fisher's highest purity) and chloroform (ACS grade) are used.

**Scope of Method.** The method for isolation of the monocarbonyl fraction from fats and oils may be divided into six steps: (1) reaction of the carbonyl compounds in the fat with 2,4-dinitrophenylhydrazine; (2) adsorption of the resulting derivatives onto activated magnesia while eliminating the bulk of the fat or oil, followed by desorption of the derivatives; (3) fractionation of the derivatives on weak alumina; (4) adsorption of the monocarbonyl derivatives on an anion exchange resin, if necessary; (5) separation of the monocarbonyl derivatives into classes on magnesia; and (6) separation of the members of each class by liquid-liquid partition chromatography.

**Step 1. Reaction with 2,4-Dinitrophenylhydrazine.** A column of Celite impregnated with dinitrophenylhydrazine, phosphoric acid, and water (reaction column) is prepared as described by Schwartz and Parks (*17*). The column is flushed with 50 ml. of benzene, followed by hexane until a colorless effluent is obtained. The fat or oil is dissolved in hexane and passed over the column.

When the last of the solution has just entered the column, the sides of the tube are washed down with hexane and the washings allowed to enter the column. Fifteen milliliters of hexane are added and permitted to drain into the column by gravity. The column is then flushed with hexane, using $N_2$ pressure until the effluent emerges colorless or has the same absorptivity (at or near 340 mμ) as the effluent from a blank column, which should always be run simultaneously.

The hexane-fat effluent now contains all of the original lipide, the 2,4-dinitrophenylhydrazine derivatives of the monocarbonyls, semialdehyde, and ketoglycerides (*7, 8*), and other classes of carbonyl compounds whose derivatives are soluble in the fat-hexane solution, a small amount of dinitrophenylhydrazine, and traces of the decomposition products of dinitrophenylhydrazine. Remaining on the column are those carbonyls whose derivatives are insoluble in the fat-hexane solution.

Quantitative Aspects. The quantitative aspects of reaction of carbonyls in fat with dinitrophenylhydrazine on the column were thoroughly studied from two standpoints. First, carbonyl compounds in the form of pure semicarbazone or other suitable derivative were added singly to monocarbonyl-free, butter oil. Schwartz (*15*) had shown that micro amounts of the semicarba-

[1] Present address, Bureau of Program Planning and Appraisal, Food and Drug Administration, Department of Health, Education, and Welfare, Washington 25, D. C.

[2] Present address, Department of Dairy Science, University of Maryland, College Park, Md.

zone and some other derivatives could be quantitatively converted to 2,4-dinitrophenylhydrazones by passing a methylene chloride–hexane solution of the derivative over a reaction column. This technique was used here, but monocarbonyl-free butter oil in hexane (25 ml. of a 14.5% solution) was substituted for hexane and the solution was run over the column at a flow rate of 46 ml. per hour. The resulting 2,4-dinitrophenylhydrazones were then isolated using Steps 2 and 3 and recoveries determined spectrophotometrically. Pertinent data are presented in Table I.

Since the results indicated that quantitative or near-quantitative recovery was obtained under a fixed set of conditions, experiments were conducted in order to ascertain whether more flexible conditions could be used without impairing the quantitative aspect. Different levels of a single batch of badly deteriorated butter oil in 50 ml. of hexane were put through Steps 1 through 3 and the isolated monocarbonyl fraction was determined spectrophotometrically. The flow rate of the solution through the column at various fat levels and the amounts of monocarbonyls found are given in Figure 1. Class separation (Step 5) of the derivatives showed that all four classes of monocarbonyls (methyl ketones, saturated aldehydes, 2-enals, and 2,4-dienals) were present in the butter oil. The near-linearity of the plot indicates that the reaction is quantitative over a wide range of fat concentration. The optimum fat concentration to use from the standpoint of throughput of solution per unit of time was calculated to be approximately 20%.

**Step 2. Adsorption of Derivatives on Magnesia.** Solvents for this and all subsequent steps need not be carbonyl-free.

The concentration of 2,4-dinitrophenylhydrazones in the effluent from Step 1 is estimated approximately by measuring the absorbancy of the solution against hexane at 340 mμ and converting the reading to micromoles, using $E = 22{,}500$.

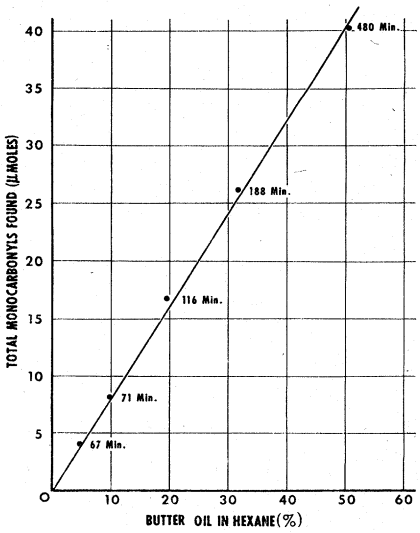


**Figure 1. Relationship between fat concentration and yield of 2,4-dinitrophenylhydrazones obtained on reaction column**

The effluent from Step 1 is carefully applied to a column of Seasorb 43–Celite 545 (1 to 1) using 1 gram of Seasorb 43 for approximately 5 to 10 μmoles of hydrazone. The column is prepared in the following manner: Equal weights of Seasorb 43 and Celite 545 are slurried in an Erlenmeyer flask with hexane so that all of the material is suspended. The slurry is then poured through a long-stemmed funnel into a chromatographic tube containing a coarse sintered-glass disk or a plug of glass wool at the constricted portion of the tube. The slurry is packed immediately under moderate air pressure (1 to 3 p.s.i.), leaving about 1 cm. of hexane above the surface of the bed. The sides of the tube are washed free of solid before the sample is applied.

The adsorbed hydrazones appear as a deep purple zone. Nitrogen pressure can be used to force the fat solution through the column, so that an unbroken stream of effluent issues from the tube continuously. No loss of hydrazones occurs from this treatment as long as colorless adsorbent can be seen. If a fat containing pigments is being investigated, the effluent may appear yellow but contains no hydrazones.

After the last of the fat solution has just entered the bed, the sides of the tube are carefully washed down with hexane. The washings are permitted to drain and a small wad of glass wool is placed just above the bed. The column is then washed with hexane until a 25-ml. aliquot of the effluent contains less than 25 mg. of lipide. Elution of the hydrazones is effected with nitromethane-chloroform (1 to 3), the effluent being collected just prior to the breakthrough of the hydrazones. Elution is continued until a colorless or very pale effluent is obtained.

The nitromethane-chloroform effluent now contains all of the monocarbonyls, semialdehyde, and ketoglycerides and probably other classes of monohydrazones. All dicarbonyl bis(2,4-dinitrophenylhydrazones), vicinal and nonvicinal, which may have been soluble in the fat-hexane effluent from Step 1 remain on the magnesia. These are manifested by blue, gray, or violet bands near or at the top of the column (see discussion). The column may have a slight violet tinge covering the area previously occupied by the hydrazones. This is due to slight decomposition of the ketoglyceride fraction. These decomposition products elute slowly with nitromethane-chloroform and account for the slight yellow tinge of the effluent, even though all of the monocarbonyls and ketoglycerides have been eluted long before.

STABILITY AND QUANTITATIVE ASPECTS. The stability and quantitative aspects of Steps 2 and 3 were determined simultaneously on 40 pure 2,4-dinitrophenylhydrazones. Approximately 0.25 μmole of each hydrazone was added singly to a 50% solution of butter oil, olive oil, or corn oil in hexane or to lard in benzene-hexane and the solution was put through Steps 2 and 3. The isolated derivative was then subjected to column partition chromatography in the chloroethanol-hexane system (Step 6) as described by Corbin, Schwartz, and Keeney (*4*). The peak was then collected and its concentration determined spectrophotometrically. Recoveries are given in Table II.

**Step 3. Fractionation of Derivatives on Weak Alumina.** The nitromethane-chloroform eluate obtained in Step 2 is evaporated on the steam bath under a stream of nitrogen until the odor of nitromethane is absent. The residue is then dissolved in hexane and applied to a column of alumina prepared as described by Schwartz and Parks (*17*). The capacity

**Table I. Recovery of Carbonyl Compounds from Butter Oil Solution after 2,4-Dinitrophenylhydrazine Reaction on Celite Column**

| Compound | Amount, μmoles | Recovery, % |
|---|---|---|
| 2-Nonadecanone (as semicarbazone) | 0.236 | 103 |
| | 2.63 | 98 |
| | 13.15 | 99 |
| 2-Octanone (as semicarbazone) | 2.20 | 101 |
| | 11.00 | 95 |
| Acetone (as ketal of glycerol) | 2.08 | 101 |
| | 10.4 | 100 |
| | 0.208 | 96 |
| Tridecanal (as semicarbazone) | 0.196 | 106 |
| | 1.96 | 103 |
| Heptanal (as oxime) | 2.38 | 98 |
| | 23.8 | 96 |
| Tetradeca-2-enal (as semicarbazone) | 0.236 | 104 |
| | 2.366 | 103 |
| | 11.83 | 99 |
| 2-Ethyl hex-2-enal (as semicarbazone) | 0.196 | 105 |
| | 1.962 | 97 |
| Octadeca-2,4-dienal (as semicarbazone) | 0.209 | 96 |
| | 2.09 | 95 |
| | 6.27 | 98 |
| Deca-2,4-dienal (as semicarbazone) | 2.48 | 93 |
| | 12.2 | 97 |

Table II. Recovery of 2,4-Dinitrophenylhydrazones from Butter Oil after Steps 2, 3, and 6

| Saturated aldehydes | Recovery, % | Methyl ketones | Recovery, % | 2-Enals | Recovery, % | 2,4-Dienals | Recovery, % |
|---|---|---|---|---|---|---|---|
| $C_2$ | 97 | $C_3$ | 95 | $C_3$ | 90 | $C_6$ | 100 |
| $C_3$ | 94 | $C_4$ | 98 | $C_4$ | 90 | $C_7$ | 96 |
| $C_4$ | 94 | $C_5$ | 98 | $C_5$ | 94 | $C_8$ | 90 |
| $C_5$ | 91 | $C_6$ | 100 | $C_6$ | 100 | $C_9$ | 95 |
| $C_6$ | 93 | $C_7$ | 95 | $C_7$ | 94 | $C_{11}$ | 97 |
| $C_7$ | 98 | $C_8$ | 99 | $C_8$ | 95 | $C_{12}$ | 98 |
| $C_8$ | 94 | $C_9$ | 94 | $C_9$ | 103 | | |
| $C_9$ | 99 | $C_{11}$ | 94 | $C_{11}$ | 90 | | |
| $C_{10}$ | 92 | $C_{13}$ | 97 | $C_{12}$ | 96 | | |
| $C_{11}$ | 90 | | | $C_{18}$ | 94 | | |
| $C_{12}$ | 101 | | | | | | |
| $C_{13}$ | 102 | | | | | | |
| $C_{14}$ | 97 | | | | | | |
| $C_{16}$ | 94 | | | | | | |
| $C_{18}$ | 102 | | | | | | |

of the alumina for hydrazones varies somewhat with the nature of the small amount of constituents eluted with the hydrazones from the magnesia in Step 2. A good rule to follow is to use 1 gram of alumina for approximately 2 to 3 μmoles present.

When the last of the hexane solution has passed into the column, the sides of the tube are washed down with a few milliliters of hexane, and the monocarbonyl fraction is eluted with a mixture of benzene-hexane (1 to 1). By using 40 model hydrazones it was established that any hydrazone in the four classes studied could be quantitatively eluted from 5 grams of alumina with 50 ml. of a 1 to 1 benzene-hexane mixture, when present at or below 8 μmoles. In practice these conditions usually hold, but in working with the carbonyl fraction isolated from a large volume of fat the ketoglyceride fraction may move too near the tail end of the monocarbonyl fraction, so that sharp fractionation is not realized. When this happens, it is recommended that about the first quarter of the ketoglyceride band be collected with the monocarbonyl fraction, the solvent removed, and the residue then rechromatographed on another column of alumina, in which case sharp fractionation of the monocarbonyl band from the ketoglyceride band is obtained. Alternatively, the column may be eluted with a weaker eluting solvent combination—e.g., benzene-hexane, 1 to 3—until all color is removed below the ketoglyceride band.

**Step 4. Adsorption of Monocarbonyl Fraction on Anion Exchanger.** Evaporation of the benzene-hexane effluent from Step 3 usually leaves a dry residue or one containing only a few milligrams of fatty impurities. This has been our experience with a number of fats and oils (butter oil, lard, and safflower, olive, and peanut oils) and it has not been necessary to resort to this step. However, in view of the large number of fats and oils that exist in nature, sufficient impurities may accompany the hydrazones to render their subsequent fractionation and identification difficult, or to complicate radioactive tracer work or gas chromatographic analyses of regenerated carbonyls. In these cases, the monocarbonyl fraction is adsorbed onto an anion exchanger in the hydroxyl form precisely as outlined by Schwartz Johnson, and Parks (*16*).

**Step 5. Separation of Monocarbonyl Fraction into Classes.** The residue from Step 3 or Step 4 (if used) is fractionated into classes, using one of the procedures described by Schwartz, Parks, and Keeney (*18*). The classes obtained are then estimated spectrophotometrically, if desired, using the proper molar extinction coefficient and maxima.

The authors have found it to their advantage when analyzing butter oil to subject the monocarbonyls from Steps 3 and 4 (if used) to partition chromatography (Step 6) prior to running the class separation. Although this involves an extra step (since one or more partition chromatograms will be run following class separation), the classes are separated more cleanly and with more confidence. The reason for this is that butter oil contains a class of naturally occurring compounds, other than the four common classes, which elutes from alumina (Step 3) with the monocarbonyl fraction. This class of constituents is relatively nonpolar, moving with the front on the partition chromatogram, and thus can be removed easily from the more polar hydrazones. The first peak from the partition chromatogram is collected by itself and later subjected to class separation. The remaining bands on the partition column are collected, pooled, and then class separated.

**Step 6. Partition Chromatography.** The classes obtained in Step 5 are subjected to column partition chromatography by the method of Corbin, Schwartz, and Keeney (*4*). The peak volume gives a good indication of the chain length of a compound, which can be confirmed by such procedures as paper chromatography, cochromatography on partition columns, ultraviolet spectra, and when enough material can be accumulated, melting point and mixed melting point.

## DISCUSSION

The procedures have been successfully used in this laboratory in the determination of carbonyl constituents of fats, oils, extracts of cheese, and whole milk powders. A number of different classes of carbonyl-containing compounds have been revealed. Among them are the ketoglycerides, a class of compounds which seem to be present in all fats and oils in various amounts and which will be discussed more fully elsewhere (*8*). The procedure also indicated early in its development that butter oil contained aldehydes bound to glycerol in phosphorus-free, acid-labile compounds; this has since been verified and the complex spectra of the aldehydes have been identified (*11*, *13*). There are indications of other classes of naturally occurring carbonyl compounds which have not yet been fully characterized.

The analysis of a fat or oil (or extract) by these procedures may not, of course, reveal the total carbonyl picture. Dicarbonyls, semialdehydes, unesterified keto acids, and other classes of compounds usually are present in autoxidized fats. Although procedures for fractionating these classes have been developed in this laboratory in conjunction with the monocarbonyl analysis, the quantitative aspects of their isolation remain to be verified.

The use of a column of Celite impregnated with 2,4-dinitrophenylhydrazine, acid, and water as a means of forming 2,4-dinitrophenylhydrazones greatly facilitated development of the subsequent steps in the procedure. The column, which was originally developed by Begemann and DeJong (*1*) and modified by Schwartz and Parks (*17*), not only gives quantitative yields of hydrazones, but also gives an acid-free solution of the derivatives, thereby eliminating the need for extraction or some other manipulation. The column procedure affords conversion of extremely low concentrations of carbonyls into the hydrazones, an achievement which apparently is difficult when the reaction is run in true solution (*3*). Thus, it was possible to identify the hydrazones of heptadecanone and octadecylaldehyde by paper chromatography after $2.4 \times 10^{-3}$ μmole (about 0.8 μg.) of the carbonyl had been put through the reaction column in 50 ml. of a 4.3% butter oil solution (in hexane) and the hydrazone subsequently isolated.

It was recognized that direct reaction of a fat with acidic 2,4-dinitrophenyl-

hydrazine might produce carbonyls from hydroperoxides, the structure which would eventually give rise to them naturally. To check this point, methyl linoleate hydroperoxide was put through the procedure. The hydroperoxide had a peroxide value of 4025 and an odor suggesting that carbonyls were present in the original preparation. It was established that the hydroperoxide was altered by passage over the reaction column, the effluent being incapable of oxidizing acidic KI. Analysis of the carbonyls obtained from 7.9 μmoles of the hydroperoxide after passage over the reaction column indicated: 0.03 μmole of ketone, 0.53 μmole of saturated aldehyde, 0.04 μmole of 2-enals, and 0.01 μmole of 2,4-dienal. To ascertain whether these carbonyls were present in the original preparation, the hydroperoxide was adsorbed from a benzene-hexane solution onto a column of Dowex 1-X4 (50- to 100-mesh) in the OH form. This procedure removed approximately 85% of the hydroperoxide, and the neutral effluent when analyzed for carbonyls gave the same data as the original hydroperoxide. It was, therefore, concluded that the original hydroperoxide was contaminated with approximately 7% of monocarbonyls and that no monocarbonyls are produced from methyl linoleate hydroperoxide on contact of this compound with the reaction column.

The acidity of the column (pH about 1.5) is sufficient to hydrolyze quantitatively and form derivatives of the aldehydes present in bound form in plasmalogens and in the phosphorus-free analog present in butter oil and in some other lipide (*5, 11, 13*). The presence of bound aldehydes in fats and oils can easily be established by applying the procedures described here on a fresh product. Although the presence of the bound aldehydes in a fat may complicate interpretation of the results of an analysis, this difficulty may be overcome by applying the procedures described by Parks, Keeney, and Schwartz (*11, 12*).

Activated magnesia has proved to be an excellent adsorbent for 2,4-dinitrophenylhydrazones. Its affinity for 2,4-dinitrophenylhydrazones is presumably due to the ionic species of the *m*-dinitro groups created by the alkaline environment. All monocarbonyl 2,4-dinitrophenylhydrazones turn various shades of red, gray, or purple on contact with this adsorbent. All of the 40 hydrazones studied were stable on magnesia and on alumina. The stability of 2,4-dinitrophenylhydrazones (also 2,4-dinitrophenylosazones) to an alkaline environment has been amply demonstrated (*14, 16, 18*).

The capacity of magnesia for 2,4-dinitrophenylhydrazones depends on factors such as fat concentration, nature of the fat under study, type of class predominantly present—i.e., ketones are held less strongly than saturated aldehydes, etc.(*18*)—and, of course, strength of the adsorbent. Very little variation in the strength of various lots of Seasorb 43 has been observed.

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